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Zooshikella harenae sp. nov., Isolated from Pacific Oyster *Crassostrea gigas*, and Establishment of *Zooshikella ganghwensis* subsp. *marina* subsp. nov. and *Zooshikella ganghwensis* subsp. *ganghwensis* subsp. nov.

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Abstract: Here, we describe the polyphasic taxonomy of a novel isolated strain WH53^T from the genus *Zooshikella* isolated from the sand sediment located between the lumen of the *Crassostrea gigas* From Germany. Phylogenetic analysis determined that the strain WH53^T had a high similarity to *Zooshikella ganghwensis* JC2044^T (99.57%) and *Zooshikella marina* LMG 28823^T (99.36%). Strain WH53^T contained ubiquinone-9 (Q-9) as the predominant menaquinone, and the major fatty acids were $C_{16:0}$, $C_{16:1}\omega7c$, and $C_{18:1}\omega7c$. Diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, amino phospholipids, and unidentified phospholipids were identified as their polar lipid composition. The DNA G+C content and genome size of strain WH53^T were 40.08 mol% and 5,914,969 bp, respectively. Digital DNA–DNA hybridisation (dDDH) for strain WH53^T against *Z. ganghwensis* JC2044^T and *Z. marina* LMG 28823^T showed low relatedness values of 26.3% and 26.1%, respectively. The extract of strain WH53^T exhibited antimicrobial property. Strain WH53^T represents a novel species in the genus *Zooshikella*. We propose the name of *Zooshikella harenae* sp. nov., with the type strain WH53^T (= DSM 111628^T = NCCB 100808^T). Furthermore, the dDDH, average nucleotide identity (ANI), percentage of conserved proteins (POCP), and amino acid identity (AAI) value between *Z. marina* LGM 28823^T and *Z. ganghwensis* DSM 15267^T were 79.9%, 97.84%, 76.08%, and 87.01%, respectively, suggesting that both of them should be reclassified as *Z. ganghwensis* subsp. *marina* subsp. nov. and *Z. ganghwensis* subsp. *ganghwensis* DSM 15267 subsp. nov.

Keywords: pacific oyster *Crassostrea gigas*; polyphasic taxonomy; Wilhelmshaven Sea; *Zooshikella*

1. Introduction

A critical reduction in discovering new antimicrobial compounds has driven us to search for rare antibiotic-producing species from underexplored habitats like the sea [1]. Microbiologists, pharmacologists, and biochemists have developed an interest in the marine environment in order to harvest water-soluble bacterial pigments. With the recent growth in awareness of the advantages of natural versus synthetic goods, the worldwide market for bio-pigments is projected to grow [2]. The analysis of a potential natural ecosystem is the first and most significant stage in locating an environment that may accommodate a wide range of bio-pigment sources. The marine environment is home to over 80% of

all living organisms [3]. It is a rich source of aquatic microbial organisms with a wider variety than their telluric equivalents [4]. The marine environment is now being touted as a promising source of bio-pigment [5]. Many microorganisms from such biotopes have previously been studied for pigment synthesis. At the same time, a large number of them are used for different industrial applications [4]. The capacity of marine microbes to create colors in harsh settings, such as very acidic/alkaline environments (pH 4 and >9), extreme temperatures (2–15 C and 60–110 °C), and restricted substrate supply, determines their choice [6,7]. In this study, we report the polyphasic taxonomy of a novel isolate from the genus *Zooshikella* from the sand sediment located between the lumen of the *Crassostrea gigas*. The genus *Zooshikella* belongs to Gram-negative bacteria, which are characterised as aerobic, chemo-organotrophic, and halophilic bacteria in the family *Zooshikellaceae* (which previously was in the family *Hahellaceae*) [8,9]. Prodigiosin and cycloprodigiosin are two members of secondary metabolites for *Zooshikella* sp. [9–11]. Many bacteria, including *Serratia marcescens*, *Hahella chejuensis*, *Streptomyces variegatus*, *Colwellia* (*Vibrio*) *psychrerythraea*, *Pseudomonas magnesiorubera*, and other eubacteria, produce prodigiosin (PDG) [12]. Prodigiosin belongs to the prodiginine community, which is made up of structural isomers with a tripyrrole center and various alkyl chains. This compound has significant biological potential because of its antimicrobial [13], antimalarial [14], anticancer [15], and immunosuppressive properties [16]. According to a recent study, prodigiosin or its analogues were recently found to be successful biological control agents against harmful algae [17,18]. They could also be used as a natural dye [19] and are classified as cell growth regulators [20]. On the other hand, cycloprodigiosin (cPDG) is only produced in trace amounts by a few marine bacteria, such as *Pseudoalteromonas* (*Alteromonas*) *rubra*, *Pseudoalteromonas denitrificans*, and *Vibrio gazogenes* [21–23]. Cycloprodigiosin hydrochloride has demonstrated potent anticancer activity against various cancer cell lines, implying that cPDG may belong to a new anticancer drug class [12,24–26]. At the moment of writing, the family of *Zooshikellaceae* is comprised of genera *Endozoicomonas*, *Kistimonas*, *Parendozoicomonas*, and *Zooshikella*. *Zooshikella* includes only two species with validly published names as *Zooshikella ganghwensis* and *Zooshikella marina* [9,11]. *Zooshikella rubidus* S1-1 has been effectively published [10], but its species name remains to obtain approval.

2. Materials and Methods

2.1. Isolation

Oysters were collected in December 2019 from the Wilhelmshaven Sea, located in the north of Germany (latitude: 53.5131; longitude: 08.14714). The strain WH53^T was isolated from the sand sediment located between the lumen of pacific oyster *Crassostrea gigas* with the dilution plate technique on the artificial seawater medium (ASW/1000 mL, agar 15.0 g, pH 7.3) from ATI Coral Ocean salt given 3.9% (*w/v*) for 12 days at 30 °C meanwhile, ASW was enriched with 2 mg/L biotin (Vitamin B7), 20 mg/L nicotinic acid, 10 mg/L thiamine (Vitamin B1), 10 mg/L 4-Aminobenzoic acid, 5 mg/L pantothenic acid, 50 mg/L pyridoxamine (Vitamin B6), 20 mg/L cyanocobalamin (Vitamin B12). To avoiding fungal contamination, 50 mg/mL cycloheximide was used. The unique pinkish-red colonies with a metallic sheen were picked up and transferred to the marine agar medium (MA) and were purified by sequential streaking on the same medium.

2.2. Morphological, Physiological, and Biochemical Studies

Morphological observation, including motility *via* light microscopy (Zeiss Axio Sc pie. A1 microscope), was conducted with cells growing on MB medium for 3 days at 30 °C. For electron microscopy, cells growing for 2 days at 30 °C in MB (marine broth) medium were fixed with aldehydes (final concentrations: 5% formaldehyde and 2% glutaraldehyde), dehydrated in a gradient series of acetone, critical point dried, and coated with gold-palladium, as previously described [27]. Images were acquired with a Zeiss Merlin field emission scanning electron microscope (FESEM) at various magnifications and a 25:75% ratio of Everhart–Thornley SE-detector and Inlens–SEM detector. Growth at

different temperatures (4, 15, 20, 25, 30, 35, 40, and 45 °C) and pH range (pH 5, 6, 7, 8, 9, 10, and 11) were evaluated on MA medium. Sodium chloride tolerance was tested using different concentration of NaCl (*w/v*): 0%, 2.5%, 5.0%, 7.5%, 10%, 15%, 25%, 30% based on the method of Kutzner [28]. A carbohydrate utilisation study was carried out on ISP9 medium supplemented with 1% carbon sources [29] and 2.5% NaCl. Incubation on all media was conducted for 9 days at 30 °C. The biochemical study was conducted by using ApiZym [30] and Api Coryne [31] stripes. Antibiotic susceptibility was tested on MA medium for 48 h containing different antibiotics: polymyxin (50 µg/mL), gentamycin (50 µg/mL), oxytetracycline (10 µg/mL), ampicillin (100 µg/mL), chloramphenicol (30 µg/mL), spectinomycin (50 µg/mL), kanamycin (50 µg/mL), cephalosporin (50 µg/mL), fusidic acid (50 µg/mL), bacitracin (50 µg/mL), thiostrepton (50 µg/mL), trimethoprim (50 µg/mL), erythromycin (15 µg/mL), and tetracycline (50 µg/mL).

2.3. 16S rRNA Gene Analysis

According to the manufacturer's instructions, genomic DNA was obtained using the Invisorb Spin Plant Mini Kit (stratec molecular, Germany). Primahana et al. [32] identified the amplification and purification of the 16S rRNA gene sequence by PCR. Primer F27 (5′AGAGTTTGATCMTGGCTCAG3′) and 1492R (5′TACGGYTACCTTGTTACGACTT-3′) [33] were used for 16S rRNA gene sequencing employing a 3730XL automatic sequencer from Applied Biosystems (ABI). The sequence was edited and assembled by BioEdit software (version 7.0.5.3) [34]. An almost-complete 16S rRNA gene sequence (1400 bp) of strain WH53^T was obtained and was deposited in GenBank with the accession number MW888978.

Identification of the closest strains of strain WH53^T based on 16S rRNA gene sequence similarity was determined using the EZBioCloud server (http://www.ezbiocloud.net/) (accessed on 12 October 2020) [35]. Phylogenetic analysis of the 16S rRNA gene was performed by uploading the 16S rRNA gene sequences of strain WH53^T in the GGDC web server (http://ggdc.dsmz.de/) (accessed on 7 October 2021) [36]. The sequence was analysed using the DSMZ phylogenomics pipeline adapted to single genes [37]. According to Meier-Kolthoff et al. [38], pairwise sequence similarities were determined. MUSCLE was used to do a multiple sequence alignment [39]. Maximum likelihood (ML) and Maximum parsimony (MP) trees were inferred by using Randomised Axelerated Maximum Likelihood (RAxML) [40] and TNT (Tree analysis using New Technology) program [41], respectively. Rapid bootstrapping with the autoMRE (extended majority rule) bootstrapping criterion [42] was employed for ML analysis. As for MP, bootstrapping replicates (1000) were used combined with tree-bisection-and-reconnection branch swapping and ten random sequence addition replicates. The sequences were evaluated using the X^2 tests employed in PAUP* (Phylogenetic Analysis using Parsimony*) [43].

2.4. Chemotaxonomy

The biomass for the chemical analyses was produced and collected for 7 days at 30 °C in a 250 mL flask containing 100 mL of MB medium on a rotary shaker (160 revolutions per minute). The freeze-dried biomass was used for the chemotaxonomic study. Minnikin's method [44] for extracting isoprenoid quinones was used and was analysed by high-performance liquid chromatography equipped with diode-array detection and mass spectrometry (HPLC-DAD-MS), as described by Risdian et al. [45] with some modification of the column, mobile phase, and flow rate. Solvent A (35% isopropanol +1% water +0.1% formic acid) and solvent B (65% acetonitrile +0.1% formic acid) were used for isocratic condition with 0.3 mL/min. A Waters ACQUITY UPLC BEH C18 column (2.1 × 50 mm, 1.7 µm) was used to separate the isoprenoid quinones. Extraction and methylation of fatty acids were done according to Sasser [46]. The analysis of fatty acid methyl esters (FAME) was conducted using Agilent 6890N gas chromatography equipped with FID (flame ionisation detector). A Macherey Nagel Optima 5 column was used to isolate the methyl esters of fatty acids (5% phenyl, 95% dimethylpolysiloxane; 50 m length; 0.32 mm

inner diameter; 0.25 μm film thickness). The retention times of individual fatty acid methyl esters were compared to standards to identify them (in-house reference standard).

2.5. Whole-Genome Analysis

Illumina's next-generation sequencing technology with MiSeq 600 cycle v3 was used for whole-genome sequencing, and Unicycler was used for genome de novo assembly [47]. Evaluation of 16S rRNA gene purity of strain WH53^T in its genome assembly was performed using the ContEst16S algorithm (https://www.ezbiocloud.net/tools/contest16s) (accessed on 12 March 2021) [48]. Automated genome annotation was carried out using the NCBI Prokaryotic Genome Annotation Pipeline PGAP [49]. The draft genome assembly was also submitted to the RAST (Rapid Annotation using Subsystem Technology) database (https://rast.nmpdr.org/) (accessed on 28 April 2021) for metabolic reconstruction analysis [50]. Prediction of secondary metabolite gene clusters was conducted using antiSMASH server (https://antismash.secondarymetabolites.org/) (accessed on 13 March 2021) [51,52]. The phylogenomic tree was created based on the whole-genome sequence of strain WH53^T and its closest phylogenetic neighbors using the Type (Strain) Genome Server (TYGS) (https://tygs.dsmz.de/) [53]. The genome sequence data of strain WH53^T were uploaded to the Type (Strain) Genome Server (TYGS) (https://tygs.dsmz.de) (accessed on 3 November 2021). The analysis also made use of recently introduced methodological updates and features [54]. The TYGS provided the results on 2021-11-02. All pairwise comparisons for phylogenomic inference were performed using Genome BLAST Distance Phylogeny (GBDP), and accurate intergenomic distances were calculated using the 'trimming' algorithm and distance formula d5 [36]. One hundred distance replicates were calculated each. A second GBDP phylogenomic analysis for generating a better resolved phylogeny was inferred using the amino acid sequences of the entire proteome [30]. The Genome-to-Genome Distance Calculator (GGDC 2.1) was used to calculate digital DDH (dDDH) values and confidence intervals using the suggested settings (GGDC 2.1) [36]. A balanced minimum evolution tree was inferred from the resulting intergenomic distances with branch support *via* FASTME 2.1.6.1, including Subtree Pruning and Regrafting (SPR) postprocessing [55]. The branch support of the tree was determined from 100 pseudobootstrap replicates each. Additionally, the KBase database was subjected to further genome-based phylogenetic analysis using the Insert Genome Into Species Tree v2.2.0 tool [56]. [56] Prokka v1.14.5 was used to annotate the assembled genome first [57]. The process was then repeated by choosing a subset of 40 public KBase genomes that were closely related to the strain genome. Alignment similarity to a subset of 49 COG (Clusters of Orthologous Groups) domains of core universal genes was used to assess relatedness. The strain genome was put into a curated multiple sequence alignment (MSA) for each COG family, concatenated, and a phylogenetic tree was built by FastTree2 using maximum-likelihood [58].

OrthoANIu algorithm [59] (https://www.ezbiocloud.net/tools/ani) (accessed on 5 May 2021) was used for the determination of average nucleotide identity (ANI), genome size, and guanine and cytosine (G+C) content. To assay genome relatedness between the strain WH53^T, the genus of *Zooshikella (Z. marina* LGM 28823^T *and Z. ganghwensis* DSM 15267^T), and the alone genus of *Mangrovitalea (M. sediminis* MCCC 1K03312^T = JCM 32104^T), besides ANI and dDDH, the average amino acid identity (AAI) value (http://enve-omics.ce.gatech.edu/aai/index) (accessed on 5 September 2021)and the percentage of conserved proteins (POCP) value (https://github.com/2015qyliang/POCP) (accessed on 9 September 2021) for analysis and interpretation of the phylogenomics pipeline from DSMZ were calculated [60]. If the POCP values are less than 50%, the species are classified into different genus, and more than 50% are in the same genus [60]. AAI less than 60% are in different genus, and more than 60% are in the same genus [61]. For this purpose, amino acid sequences from POCP for AAI were used. Draft genome of strain WH53^T and *Zooshikella marina* LMG 28823^T were deposited at DDBJ/EMBL/GenBank under the accession number JAGSOY000000000 and JAGSGA000000000, respectively.

2.6. DNA–DNA Hybridisation (DDH)

DNA–DNA hybridisation was performed following the procedure of Ziemke et al. [62], except that for nick translation, 2 µg DNA was labeled during 3 h of incubation at 15 °C. This method was performed for the DNA of strain WH53^T and its closest relatives, *Zooshikella ganghwensis* DSM 15267^T and *Zooshikella marina* LGM 28823^T.

2.7. Secondary Metabolite Production and Antimicrobial Activity

The strain WH53^T was grown in 250 mL Erlenmeyer flasks containing 100 mL MB medium containing 2% (*v/w*) XAD-2 polymeric resin for 5 days at 30 °C on a shaker (160 revolutions per minute). Separation of XAD-2 was conducted by filtration, and acetone was used to extract secondary metabolites from XAD-2. The extract was dried by a rotary evaporator (40 °C). The dried extract was dissolved with 1 mL methanol, which resulted in the initial value of concentration of 100%. The extract was tested against some microbes: *Escherichia coli* wild type BW25113, *Escherichia coli* acrB JW25113, *Pseudomonas aeruginosa* DSM 19882, *Staphylococcus aureus* Newman, *Citrobacter freundii* DSM 30039, *Acinetobacter baumannii* DSM 30008, *Bacillus subtilis* DSM 10, *Mycobacterium smegmatis* ATCC 700084, *Mucor hiemalis* DSM 2656, *Wickerhamomyces anomalus* DSM 6766, and *Candida albicans* DSM 1665. The MIC (minimal inhibitory concentration) values were determined in 96-well microplates by incubating the bacterial test strain and the extract in Mueller–Hinton (MH) broth (Merck, Darmstadt, Germany) and Middelbrock broth medium (for *M. smegmatis* ATCC 700084; Becton, Dickinson and Company, Le Pont-de-Claix, France) with starting OD600 = 0.01. For the fungal test strain, the initial OD600 = 0.05 and Mycosel broth (Myc; Carl Roth GmbH + Co. KG, Karlsruhe, Germany; 1% phytone peptone, 1% glucose, 50 mM HEPES) was used for the evaluation. The lowest concentration of the tested extract that inhibited the observable growth of test microorganisms was defined as the MIC value. Twofold serial dilutions were used to determine the antimicrobial activity of the extract (6.67–0.05%) [63].

3. Result and Discussion

3.1. Morphological, Physiological, and Biochemical Results

Strain WH53^T was a motile, spirillum form; cell size measurement demonstrated a diameter of 0.6–0.7 µm in width and 4.1–13.5 µm in length (Figure 1) and no-spore-form. The optimal growth temperature was observed at 30 °C, and the pH optimum was revealed at pH 7. Growth was not detected on the medium without NaCl. Sodium chloride tolerance was up to 7.5%, with the optimum growth on media containing 2.5% NaCl. Study on biochemical property-based API ZYM test suggested that strain WH53^T had the strong activity of phosphatase alkaline and leucine arylamidase, but no activity for chymotrypsin and β-glucuronidase (Table 1). The extract of strain WH53^T could strongly inhibit *Escherichia coli* wild type BW25113, *Escherichia coli* acrB JW25113, *Staphylococcus aureus* Newman, *Citrobacter freundii* DSM30039, *Acinetobacter baumannii* DSM 30008, *Bacillus subtilis* DSM 10, *Mycobacterium smegmatis* ATCC 700084, *Mucor hiemalis* DSM 2656, *Wickerhamomyces anomalus* DSM 6766, and *Candida albicans* DSM 1665. Moderate activity was only found against *Pseudomonas aeruginosa* DSM 19882 (Table S1). This result suggested that strain WH53^T could produce active compounds against various microbes that might benefit oysters to protect themselves from pathogenic microbes.

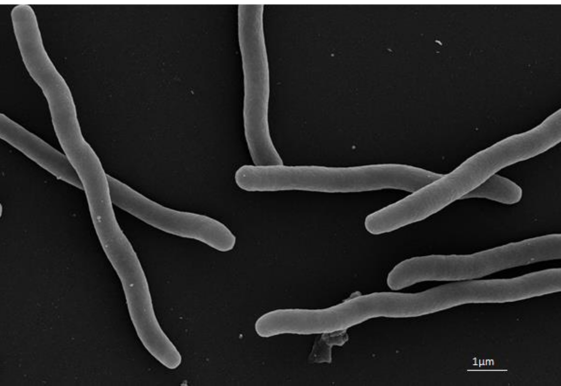

Figure 1. Scanning electron microscopy images of strain WH53^T.

Table 1. Enzymes produced by strain WH53^T detected by ApiZym system.

Enzyme	Observation	Enzyme	Observation
Phosphatase alkaline	++	Naphtol-AS-BI-phosphohydrolase	++
Esterase (C4)	+	α-galactosidase	(+)
Esterase lipase (C8)	+	β-galactosidase	(+)
Lipase (C14)	(+)	β-glucuronidase	-
Leucin arylamidase	++	α-glucosidase	++
Valine arylamidase	(+)	β-glucosidase	(+)
Cystine arylamidase	(+)	N-acetyl-beta- glucosaminidase	++
Trypsin	(+)	α-mannosidase	+
Chymotrypsin	-	α-fucosidase	(+)
Phosphatase acid	++		

++ strong; + good; (+) weak; - no activity.

Strain WH53^T was sensitive to polymyxin, gentamycin, chloramphenicol, spectinomycin, kanamycin, erythromycin, and tetracycline. Resistance was detected against oxytetracycline, ampicillin, cephalosporin, fusidic acid, bacitracin, thiostrepton, and trimethoprim.

3.2. 16S rRNA Gene Analysis

Based on the result from the EZBioCloud server, strain WH53^T had high sequence similarities to *Zooshikella ganghwensis* JC2044^T (99.57%) and *Zooshikella marina* JC333^T (99.36%). Strain WH53^T was also closely related to '*Zooshikella rubidus*' S1-1 (99.36%). The results of phylogenetic analyses showed that strain WH53^T formed a clade with *Z. ganghwensis* JC2044^T, *Z. marina* JC333^T, and '*Z. rubidus*' S1-1 supported by bootstrap values of 100% from ML and MP analyses (Figure 2).

3.3. Chemotaxonomic Characterization

Major detected fatty acids of strain WH53^T were $C_{16:0}$ (39.01%), $C_{16:1}\omega7c$ (41.12%), and $C_{18:1}\omega7c$ (11.31%). Diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), aminophospholipid (APL), and unidentified phospholipids (PL) were the polar lipid identified in strain WH53^T (Supplementary Figure S1). The major quinone of strain WH53^T was ubiquinone-9 (Q-9).

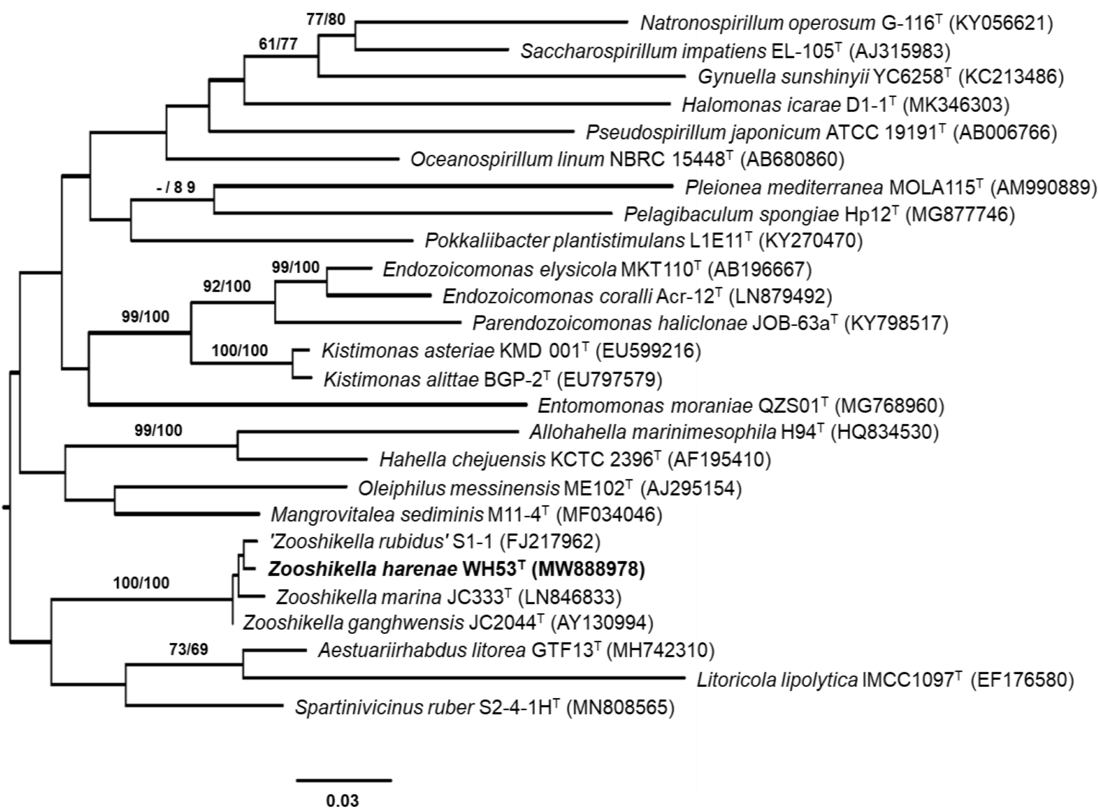

Figure 2. The GTR+GAMMA model was used to infer the ML tree, which was then rooted using midpoint-rooting. The branches were scaled in terms of the expected number of substitutions per site. The numbers above the branches represent the support values obtained from ML (left) and MP (right) bootstrapping. They were meaningful more than 60%. After 1000 replicates, the ML bootstrapping converged, with an average support of 59.57%. MP analysis yielded the best score of 1942 (consistency index 0.41, retention index 0.44) and two best trees. The MP bootstrapping average support was 64.04%.

3.4. Genomic Characteristics and Phylogenomic Analysis

Only one 16S rRNA gene sequence was detected in the whole-genome sequence of strain WH53^T, indicating that the genomic data was not contaminated by other organisms. The draft genome assembly of strain WH53^T consisted of 5,914,969 bp (377 contigs) with a G+C content of 40.08 mol% (GenBank accession No. JAGSOY000000000). The phylogenomic tree with the very low average branch support (25.8%) derived from TYGS using genome sequence showed that strain WH53^T, instead of being close to *Z. ganghwensis* DSM 15267^T and *Z. marina* LMG 28823^T, was located in a low supported clade with *Azomonas agilis* DSM 375^T (Supplementary Figure S2). On the other hand, the phylogenetic tree based on whole-proteome-based GBDP distances showed a very high average branch support of 94.1%, which is more reliable than the phylogenomic tree result (Figure 3). Based on the proteome tree, strain WH53^T was in a very high supported clade together with *Z. ganghwensis* DSM 15267^T and *Z. marina* LMG 28823^T. The other phylogenomic tree resulted from the Insert Genome Into Species Tree v2.2.0 tool [56] using 49 COG suggested that strain WH53^T was in one clade together with other *Zooshikella* species with very high supporting value (Figure 4).

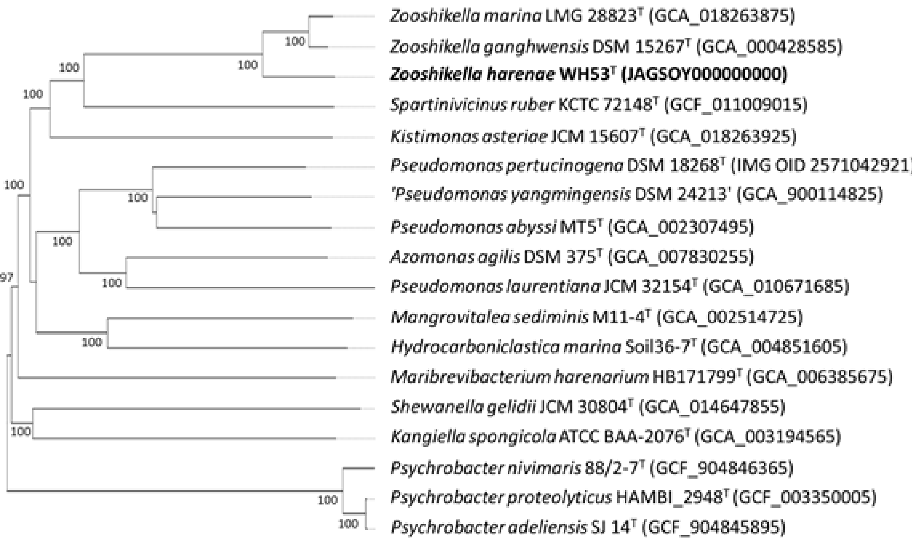

Figure 3. Phylogenomic tree inferred from whole-proteome-based GBDP distances showing relationships between strain WH53^T and the closely related type strains based on Type (Strain) Genome Server (TYGS) (https://tygs.dsmz.de/) (accessed on 3 November 2021) [54]. The number at the nodes are GBDP pseudo-bootstrap support values >60% from 100 replications, with average branch support of 94.1%.

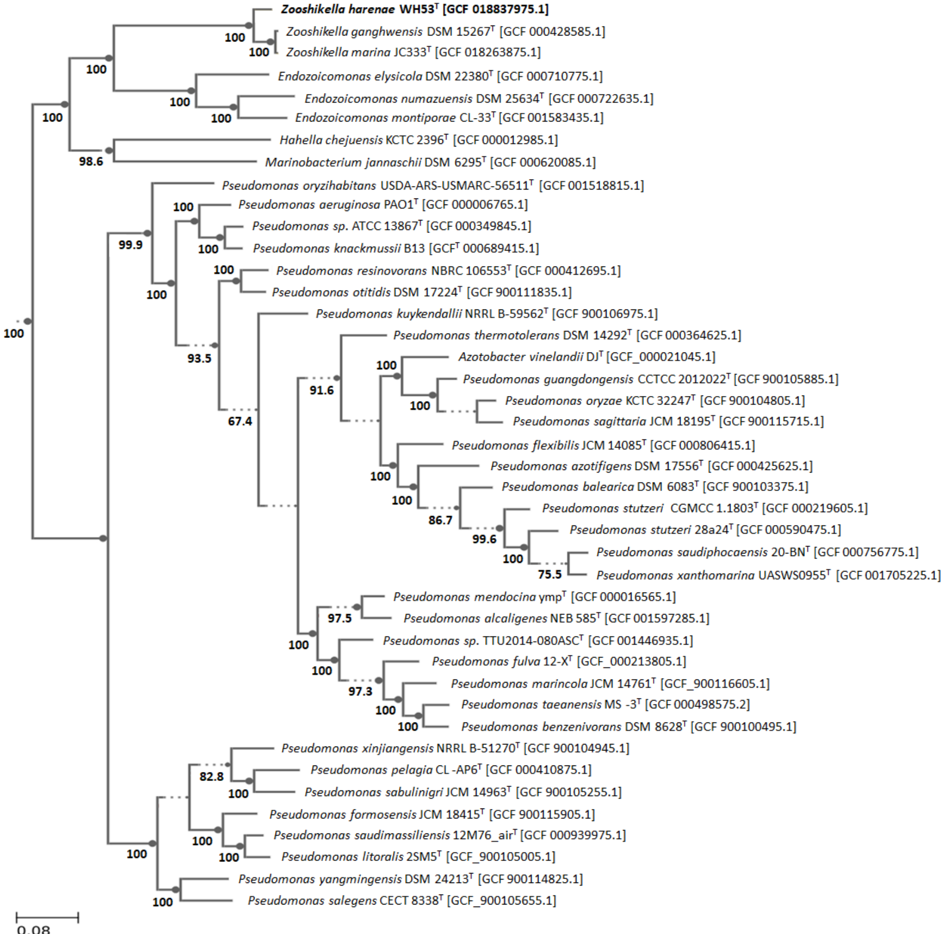

Figure 4. Phylogenomic tree showing relationships between strain WH53^T and the closely related type strains based on Insert Genome into Species Tree v2.2.0 tool [56] using 49 COG (Clusters of Orthologous Groups) domains of core universal genes. Bootstrap values >60% from 1000 replications are displayed at the nodes.

As shown in Table 2, all of the compared type strains had ANI values lower than the species cut-off value of 95%, and dDDH scores were below the 70% threshold value [64]. The DNA–DNA hybridisation (DDH) analysis of strain WH53^T and its closest relatives revealed a value of 50.1% against *Z. ganghwensis* DSM 15267^T and 34.6% against *Z.marina* LGM 28823^T, which are also below the 70% cut-off point for species delineation [65]. However, the dDDH score between *Z. marina* LGM 28823^T and *Z. ganghwensis* DSM 15267^T was more than 70%, indicating that they belong to the same species. Meier-Kolthoff [37] suggested that a value of 79–80% dDDH was the threshold for delineating subspecies.

Table 2. ANI and dDDH values of the strain WH53^T and its closely related type strains.

Strain	***Zooshikella harenae* WH53^T (JASOY000000000)**		***Zooshikella marina* LMG 28823^T (JAGSGA000000000)**	
	OrthoANIu (%)	**dDDH (%)**	**OrthoANIu (%)**	**dDDH (%)**
***Zooshikella harenae* WH53^T (JAGSOY000000000)**	100	100	82.74	26.10
***Zooshikella marina* LMG 28823^T (JAGSGA000000000)**	82.74	26.10	100	100
***Zooshikella ganghwensis* DSM 15267^T (AUAF01000000)**	82.78	26.30	97.84	79.90
***Endozoicomonas montiporae* CL-33^T (CP013251)**	66.94	24.30	66.76	28.50
***Endozoicomonas elysicola* (JOJP01000000)**	66.93	27.50	67.44	23.30
***Endozoicomonas arenosclerae* (LASA01000000)**	66.88	23.10	67.26	22.30
***Endozoicomonas atrinae* (LUKQ02000000)**	67.25	23.50	67.17	22.50
***Hahella chejuensis* KCTC 2396^T (CP000155)**	66.79	38.60	66.43	29.00
***Marinobacter lutaoensis* (MSCW01000000)**	66.03	23.20	65.40	21.50
***Mangrovitalea sediminis* (NTLB01000000)**	66.08	32.80	65.95	30.80
***Aestuariirhabdus litorea* (QWEZ01000000)**	66.59	19.30	66.88	18.60
***Kistimonas asteriae* JCM 15607^T (JAEVHF000000000)**	66.84	20.00	67.08	34.40
***Azomonas agilis* DSM375^T (NZ_VLKG00000000)**	67.62	31.80	66.35	20.40
***Hydrocarboniclastica marina* Soil36-7^T (PRJNA479718)**	68.07	38.60	68.17	39.50
***Kangiella spongicola* ATCC BAA-2076^T (PRJNA473557)**	67.74	32.90	67.63	34.10
***Spartinivicinus ruber* KCTC 72148^T (PRJNA607118)**	68.91	21.30	69.06	22.80

OrthoANIu values ≥95% and dDDH values >70% are shaded gray.

Some phenotypic similarities exist between *Z. marina* LGM 28823^T and *Z. ganghwensis* DSM 15267^T, such as the same NaCl tolerance (5%) and some enzymatic activities. The genome was predicted to contain a total of 5214 genes, comprising 5180 protein-coding

genes, 54 tRNA genes, 3 rRNA genes, and 4 non-coding RNA (Table 3). Both types of strains had no activity for myristate lipase, cystine arylamidase, trypsin, naphtol-AS-BI-phosphohydrolase, α-Glucosidase, β-Glucosidase, and α-fucosidase. Since the dDDH value between these two strains was 79.9%, and they had some similar phenotypic characteristics, therefore, *Z. marina* LGM 28823^T could be reclassified as the subspecies of *Z. ganghwensis*. Moreover, based on the 60% AAI value [61] and the 50% POCP value [60] proposed as genus boundaries, strain WH53^T could be classified within the genus *Zooshikella* (Table 4).

Table 3. Comparison of phenotypic characteristics that distinguish strain WH53^T from the most closely related *Zooshikella* species. Strains: 1, WH53^T; 2, *Zooshikella marina* LMG 28823^T; 3, *Zooshikella ganghwensis* DSM 15267^T; 4, *Zooshikella rubidus* S1-1^T.

Characteristic	1	2	3	4 †
pH range for growth	6–8	6–9	5–8	4.5–9.5
NaCl tolerance (*w*/*v*)	7.5%	5.0%	5.0%	10%
Color of colony	Red	Red	Red	Dark red
Butyrate esterase (C4)	+	(+)	(+)	-
Caprylate esterase lipase (C8)	+	(+)	(+)	+
Myristate lipase (C14)	(+)	-	-	-
Cystine arylamidase	(+)	-	-	-
Trypsin	(+)	-	-	-
Acid phosphatase	++	(+)	+	+
Naphtol-AS-BI-phosphohydrolase	++	-	-	-
α-Glucosidase	+	-	-	-
β-Glucosidase	(+)	-	-	-
N-acetyl-β-glucosaminidase	++	++	-	-
Phosphatase alkaline	++	++	++	+
Valine arylamidase	(+)	(+)	(+)	-
α-galactosidase	(+)	-	-	-
β-galactosidase	(+)	-	-	-
β-glucuronidase	-	-	-	-
α-mannosidase	+	+	-	-
α-fucosidase	(+)	-	-	-
Glucose fermentation	-	+	-	NR
Sucrose fermentation	-	+	-	NR
Glycogen fermentation	-	+	-	NR
Polar lipids	DPG–PG–PE–APL–PL	DPG–PG–PE–APL–PL	DPG–PG–PE–AL–PL	NR
Major fatty acid	$C_{16:0}$ $C_{16:1}\omega7c$ $C_{18:1}\omega7c$	$C_{12:0}$ 3-OH $C_{14:0}$ $C_{16:0}$ $C_{16:1}\omega7c$ $C_{18:1}\omega7c$	$C_{12:0}$ 3-OH $C_{16:0}$ $C_{16:1}\omega7c$ $C_{18:1}\omega7c$	$C_{12:0}$ 3-OH $C_{16:0}$ $C_{18:1}\omega7c$
Major quinone	Ubiquinone-9	Ubiquinone-9	Ubiquinone-9	Ubiquinone-9
Ampicillin susceptibility	-	NR	NR	-
Tetracycline susceptibility	+	NR	NR	-
Total sequence length (bp)	5,914,969	6,060,265	5,798,664	NR
Contigs	377	83	414	NR
No. of protein	5,120	5,083	4,943	NR
rRNA	3	3	10	NR
tRNA	54	55	55	NR
No. of Gene	5241	5160	5164	NR
Other RNA	4	4	4	NR
Pseudogene	60	15	152	NR
G + C content (mol%)	40.08%	40.94%	41.02%	41%

++ strong; + good; (+) weak; - no activity; NR not reported. † Data from Lee et al. [10]. Diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), aminophospholipid (APL), and unidentified phospholipids (PL).

Table 4. Genome relatedness between the strain WH53^T and the other related type strains according to the average amino acid identity (AAI) value and the percentage of conserved proteins (POCP) value. Strains: 1, *Zooshikella ganghwensis* DSM 15267^T (AUAF01000000); 2, *Zooshikella marina* LMG 28823^T (JAGSGA000000000); 3, *Zooshikella harenae* WH53^T (JAGSOY000000000).

Strain	1		2		3	
	POCP (%)	AAI (%)	POCP (%)	AAI (%)	POCP (%)	AAI (%)
Zooshikella harenae WH53^T (JAGSOY000000000)	76.08	87.01	77.37	86.73	100	100
Zooshikella marina LMG 28823^T (JAGSGA000000000)	76.08	87.01	100	100	77.37	86.73
Zooshikella ganghwensis DSM 15267^T (AUAF01000000)	100	100	76.08	87.01	76.08	87.01
Mangrovitalea sediminis (= MCCC 1K03312^T = JCM 32104^T) (NTLB01000000)	29.76	49.90	30.51	49.76	31.26	49.79
Endozoicomonas acroporae strain Acr-14^T (PRJNA422318)	28.44	51.65	28.40	51.37	28.58	51.20
Endozoicomonas arenosclerae (PRJNA279233)	29.50	50.97	29.08	50.74	29.40	50.58
Endozoicomonas ascidiicola (NZ_LUTW00000000)	30.17	51.00	30.26	51.06	29.82	51.32
Endozoicomonas atrinae (NZ_LUKQ00000000)	26.35	50.80	26.53	50.72	26.32	50.72
Endozoicomonas elysicola (NZ_JOJP00000000)	30.89	51.22	31.17	51.11	31.27	51.19
Endozoicomonas montiporae Strain LMG 24815^T (NZ_JOKG00000000)	30.03	51.25	30.26	51.24	29.86	51.37
Endozoicomonas numazuensis (NZ_JOKH00000000)	29.82	51.41	29.94	51.11	30.08	51.02
Hahella chejuensis KCTC 2396^T (PRJNA16064)	30.68	49.72	31.48	49.62	31.44	49.73
Hahella ganghwensis DSM 17046^T (NZ_AQXX00000000)	30.01	49.56	30.54	49.41	30.67	49.17
Kistimonas asteriae (NZ_JAEVHF000000000)	33.61	53.09	33.94	52.56	34.09	52.75
Parendozoicomonas haliclonae (NZ_FWPT00000000)	31.66	51.49	32.19	51.20	32.17	50.91
Pseudomonas xiamenensis (NZ_JACLGO000000000)	30.30	51.18	30.28	51.06	30.28	50.89
Kistimonas asteriae JCM 15607 ^{T} (JAEVHF000000000)	33.61	53.09	33.92	52.55	34.09	52.75
Spartinivicinus ruber KCTC 72148^T (PRJNA607118)	49.90	58.67	49.94	58.38	49.97	58.69
Pseudomonas pertucinogena JCM 11590^T (BMNN00000000)	32.11	52.18	32.53	52.10	32.18	51.93
Pseudomonas yangmingesis DSM 24213^T (NZ_FOUI00000000)	31.78	52.27	32.05	52.20	31.74	51.95
Pseudomonas abyssi Strain MT5^T (PRJNA406957)	31.34	51.28	32.07	51.16	31.90	50.99
Azomonasa gilis DSM 375^T (NZ_VLKG00000000)	27.95	50.87	28.25	51.03	28.53	50.95
Hydrocarboniclastica marina Soil36-7^T (PRJNA479718)	28.46	49.31	28.65	49.18	28.97	49.10
Kangiella spongicola ATCC BAA-2076^T (PRJNA473557)	25.06	47.38	25.60	47.41	24.74	47.11

POCP less than 50% is a different genus, and more than 50% is the same genus AAI less than 60% is a different genus, and more than 60% is the same genus.

It also revealed that 22% of the genes were assigned to subsystems (Figure 5). Amino acid and derivatives metabolism had the largest feature counts (307), followed by protein metabolism (223) and carbohydrates metabolism (191). Genes responsible for motility and chemotaxis (100), metabolism of aromatic compounds (14), and stress response (76) were also detected. However, only two genes were observed for dormancy and sporulation. The antiSMASH server predicted 18 clusters of genes involved in secondary metabolite biosynthesis, with three clusters demonstrating higher than 60% similarity to known biosynthetic gene clusters: althiomycin biosynthetic gene cluster (62%), indigoidine biosynthetic gene cluster (80%), and ectoine biosynthetic gene cluster (83%).

Besides the result of genotypic studies such as 16S rRNA gene analysis, ANI, dDDH, and DDH, strain WH53^T could also be delineated from its closely related type strains by several phenotypic characteristics (Table 3). Myristate lipase, cystine arylamidase, and trypsin were detected in strain WH53^T, while the other *Zooshikella* type strains had no activity for them. Sodium chloride tolerance of strain WH53^T was higher than the other *Zooshikella* type strains.

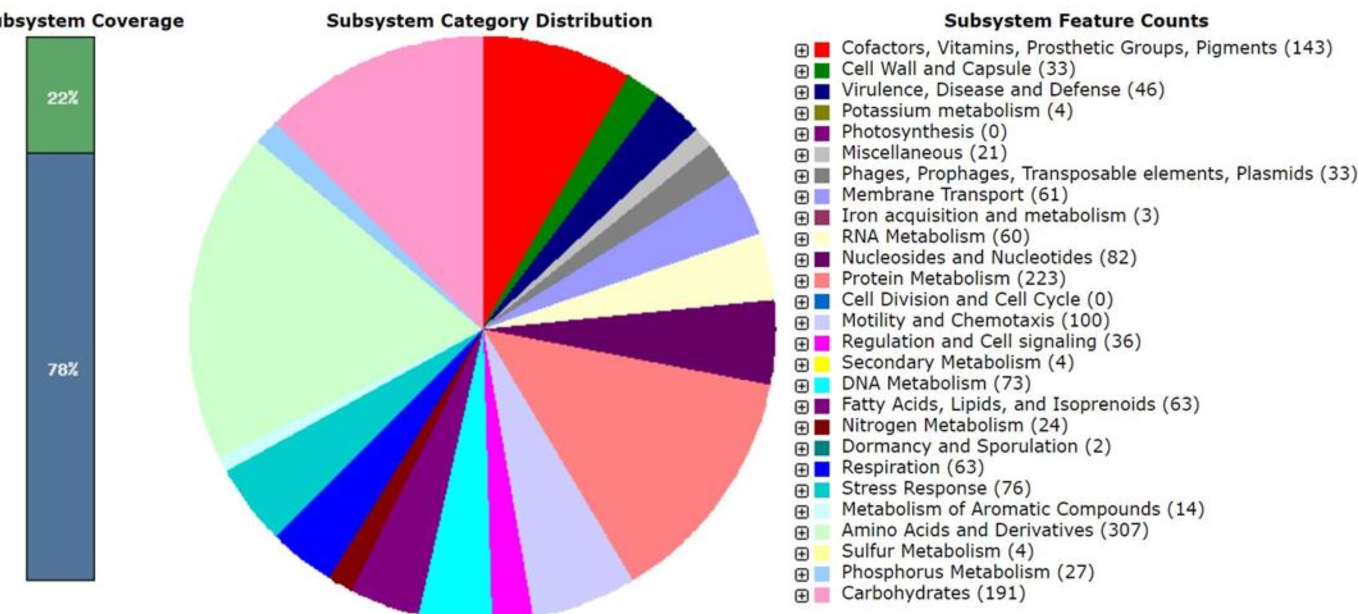

Figure 5. Subsystem category distribution of strain WH53^T based on RAST annotation server (https://rast.nmpdr.org/) (accessed on 28 April 2021).

4. Conclusions

Based on comparing all phenotypic and genotypic traits, strain WH53^T belongs to genus *Zooshikella* and can be separated from the other *Zooshikella* species as a novel species in the genus *Zooshikella*, for which the name *Zooshikella harenae* sp. nov. is proposed.

Description of *Zooshikella harenae* sp.nov.

Zooshikella harenae (ha.re′nae. L. gen. n. harenae of sand, referring to the isolation of the strain from the sand trapped in the pacific oyster *Crassostrea gigas*): Optimal growth is observed at 30 °C, pH 7 and 2.5% (*w/v*) NaCl. Sodium chloride is required for growth. Grows on MA as circular, convex, entire, glistening, opaque, and viscid colonies are pinkish-red colonies with a metallic sheen and approximately 0.5 mm in diameter. Cells are Gram-stain-negative, motile, spirillum form with aerobic and non-spore-forming. Large amounts of red pigment with a metallic green sheen are produced on an agar medium. Strong activity in acid phosphatase, alkaline phosphatase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, N-acetyl-beta-glucoseamidase. Good activity for esterase (C4), esterase lipase (C8), α-mannosidase. Weak activity for lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-galactosidase, β-galactosidase, β-glucosidase, α-fucosidase, and no activity for β-Glucoronidase and chymotrypsin. Glucose, mannose, fructose are used as sole carbon sources, but not arabinose, sucrose, xylose, inositol, rhamnose, raffinose, and cellulose. Major fatty acids are $C_{16:0}$ (39.01%), $C_{16:1}\omega7c$ (41.12%), and $C_{18:1}\omega7c$ (11.31%). Diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), amino phospholipids (APL), and unidentified phospholipids (PL) are identified as the polar lipid. The major quinone is ubiquinone-9 (Q-9). The type strain is WH53^T (= DSM 111628^T = NCCB 100808^T), isolated from pacific oyster *Crassostrea gigas* collected from the Whilmshaven, Germany. DNA G+C content is 40.08 mol%. The genome size is 5,914,969 bp with 5120 coding sequences, 54 tRNA genes, and three rRNA operons. The complete genome and the 16S rRNA sequence of strain WH53^T were deposited at NCBI GenBank with accession numbers JAGSOY000000000 and MW888978, respectively.

Description of *Zooshikella ganghwensis* subsp. *marina* subsp. nov.

Zooshikella ganghwensis subsp. *marina* (ma.ri'na. L. fem. adj. *marina* of the sea, marine): Basonym: Zooshikella marina Ramaprasad et al. 2015.

The description is identical to that given for *Zooshikella marina* by Ramaprasad et al. [11]. Type strain is JC333^T (=KCTC 42659^T = LMG 28823^T).

Description of Zooshikella ganghwensis subsp. ganghwaensis subsp. nov.

Zooshikella ganghwensis (gang.hwen'sis. N.L. fem. adj. *ganghwensis*, Ganghwa Island, Korea):

Basonym: Zooshikella ganghwensis Yi et al. 2003.

The description is identical to that given for *Zooshikella ganghwensis* by Yi et al. [9]. The type strain is JC2044^T (=IMSNU 14003^T = KCTC 12044^T = DSM 15267^T), and the subspecies was created on the basis of rule 40d of the Bacteriological Code [66].

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/d13120641/s1, Figure S1: Two-dimensional thin-layer chromatogram of polar lipids extracted from (a) strain WH53T; (b) Zooshikella marina LMG 28823T; (c) Zooshikella ganghwensis DSM 15267T. Figure S2: Phylogenomic tree inferred from GBDP distances calculated from genome sequences showing relationships between strain WH53T and the closely related type strains based on Type (Strain) Genome Server (TYGS) (https://tygs.dsmz.de/) (accessed on 3 November 2021). The number at the nodes are GBDP pseudo-bootstrap support values > 60% from 100 replications, with average branch support of 25.8%. Table S1: Antimicrobial activity of Strain WH53T against human pathogens shown in MIC value (%).

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Data Availability Statement: The GenBank accession number for the 16S rRNA gene sequence of strain WH53^T is MW888978. Draft genome of strain WH53^T and *Zooshikella marina* LMG 28823^T were deposited at NCBI/GenBank under the accession number JAGSOY000000000 and JAGSGA000000000, respectively.

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